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- Carcy, Bernard  
34070 Montpellier (FR)
- Gorenflot, André  
34090 Montpellier (FR)
- Précigout, Eric  
34730 Prades Le Lez (FR)
- Vallet, Alexina  
St. Paul en Chablais (FR)

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(71) Applicant: Akzo Nobel N.V.  
6824 BM Arnhem (NL)

(74) Representative:  
Mestrom, Joannes Jozef Louis et al  
N.V. Organon,  
Postbus 20  
5340 BH Oss (NL)

(72) Inventors:

- Schetters, Theodorus Petrus Maria  
5432 CD Cuyk (NL)

(54) **Babesia vaccine**

(57) The present invention relates to the nucleic acid sequence of genes encoding proteins of the 37 kD protein family of *Babesia divergens*. Furthermore, the invention relates to recombinant molecules comprising such nucleic acid sequences, live recombinant carriers comprising such nucleic acid sequences and host cells comprising such nucleic acid sequences. Yet another embodiment of the invention relates to vaccines against *Babesia divergens* comprising proteins of the 37 kD protein family, nucleic acid sequence of genes encoding proteins of the 37 kD protein family of *Babesia divergens*, recombinant molecules comprising such nucleic acid sequences, live recombinant carriers comprising such nucleic acid sequences, host cells comprising such nucleic acid sequences, antibodies against proteins of the 37 kD protein family and methods for the preparation of such vaccines. Additionally, diagnostic kits for the detection of *Babesia divergens*, diagnostic kits for the detection of antibodies against *Babesia divergens* and methods for the detection of *Babesia*

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## Description

[0001] The present invention relates to the nucleic acid sequence of genes encoding proteins of the 37 kD protein family of *Babesia divergens*: recombinant molecules comprising such nucleic acid sequences, live recombinant carriers comprising such nucleic acid sequences, host cells comprising such nucleic acid sequences, vaccines against *Babesia divergens* comprising proteins of the 37 kD protein family, methods for the preparation of such vaccines, methods for the detection of antibodies against a protein of the 37 kD protein family, diagnostic kits for the detection of *Babesia divergens*, diagnostic kits for the detection of antibodies against *Babesia divergens* and methods for the detection of *Babesia divergens*.

[0002] The parasite *Babesia divergens* causes severe economical losses in cattle. The genus *Babesia* to which *Babesia divergens* belongs comprises amongst others also the species *B. bovis*, *B. canis* and *B. bigemina*. In Europe, *Babesia divergens* is the most pathogenic *Babesia* species affecting cattle. (Kuttler, K.L., in M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, Fla. 1988). The parasite is transmitted by the three-host cattle-tick *Ixodes ricinus*. *Babesia divergens* causes anaemia in cattle, in severe cases leading to death. The disease is also called "red water" since in a progressed state it causes bloody urine. The problem of bovine Babesiosis has been a long standing problem: vaccination against bovine Babesiosis in Sweden, using blood from carrier cattle which had acute Babesiosis in the previous summer, was already advised by local veterinary authorities in 1920. In spite of all efforts however, there is still no safe and efficacious vaccine commercially available today, although in Sweden alone, babesiosis costs farmers more than 875.000 \$ each year. It has been shown that babesiosis in cattle can be controlled by vaccination with live attenuated vaccines. The presently available live vaccines however have the drawback that their application needs veterinary surveillance. This is due to their variable infectivity and morbidity: in unhealthy animals an attenuated live vaccine causes a virulent infection leading to sickness.

It is known that vaccination with live vaccines is however not necessary. It appeared that so-called Soluble Parasite Antigen (SPA) preparations are capable of inducing an immune response that, although not necessarily affecting the parasite, sufficiently reduces the level of clinical manifestations upon infection. (Rev.: Schetters et al. in Parasitology today 11: 456-462 (1995)).

Further analysis of the various components found in SPA has revealed several proteins that possibly play a role in inducing protection against infection, or at least against the clinical manifestations of the infection. When the SPA proteins were separated into 4 separate groups F1-F4, based on their molecular weight, it surprisingly turned out that all 4 groups contained a compound that gave at least a certain amount of protection against infection. (Précigout et al., Experimental Parasitology 77: 425-434 (1993)).

Of the proteins found in the F4-fraction, one specific *Babesia divergens* protein has been studied for several years already for its use in vaccines: the 17 kD Merozoite Membrane protein. Of this protein it has been strongly suggested that it plays a very important role, "not the key role, in inducing immunity against *Babesia*. This was based i.a. upon the finding that monoclonal antibodies against the 17 kD protein were induced after live infection and could *in vitro* drastically inhibit growth of the parasite. (Précigout et al. (1993), Exp. Parasitol. 77 (4): 425-34).

[0003] Two other proteins, present in the same fraction that comprises the 17 kD protein, i.e. the F4-fraction, and also recognised by antiserum against *Babesia divergens*, are a 50 kD protein and a 37 kD protein. (Carcy et al., Infect. and Immunity. 63: 811-817 (1995)). The detection of both the 50 kD and the 37 kD protein was based on the fact that *in vivo* antibodies are raised against these proteins. This is however the case with many proteins of the SPA: practically all proteins are capable of raising antibodies, but only one or a minor number of proteins plays a role in the induction of neutralising antibodies.

Only antibodies against the 17 kD protein were known to possess protective activity. Thus, one would expect this 17 kD protein to be the protein of the F4 group that is responsible for inducing immunity. And therefore the possible role of the other F4 proteins, e.g. the 37 kD and the 50 kD protein remained fully unclear.

[0004] It is an objective of the present invention to provide a novel and efficient vaccine component against *Babesia divergens* infection in cattle, or at least against the clinical manifestations of the infection.

[0005] In one embodiment, therefore, the present invention provides for the first time the nucleotide sequence of the gene encoding the 37 kD protein. Surprisingly it was found, that the 37 kD protein can be found in at least two different variant forms: a 37 kD form and a slightly shorter form, a 35 kD form. Other, slightly smaller or larger forms are also seen. The form depends on the *Babesia divergens* strain from which the gene was isolated. It is therefore appropriate to talk about a 37 kD protein family rather than merely a 37 kD protein. The reason for the difference in size of the various members lies in the fact that certain regions of the protein can apparently be deleted without drastically altering the function of the protein (see below). Many different *Babesia divergens* strains have until now been isolated

fragment that is present in one protein and deleted in the other contributes to a decrease in homology level, even in the case that all nucleic acids still present would be 100 % homologous. The following may serve as an example: if the genes encoding a 37 kD protein and a 35 kD protein are compared, there is a non-homology of 5% which is only due to the deletion in the shorter of the two genes. Therefore, in order to compensate for those differences in homology that are due to differences in size of the proteins of the 37 kD protein family, the minimal level of overall homology between the genes encoding the various proteins of the 37 kD protein family is estimated to be 60%. Thus, the 37 kD protein family comprises those proteins of which the genes encoding them share at least 60% homology with the genes encoding the 37 kD protein family of which the nucleic acid sequences are given in SEQ ID NO: 1, 2 and 3. These sequences are merely given as examples of proteins of the 37 kD protein family. The algorithm used for the determination of the level of nucleic acid homology is known as "Clustal W" and has been described by Thompson et al., in Nucleic Acid Research 22: 4673-4680 (1994). The program can be found at several sites on Internet.

All genes encoding a protein of the 37 kD protein family that share at least 60% homology with the nucleic acid sequences given in SEQ ID NO: 1, 2 or 3 are considered to be members of the 37 kD protein family. For e.g. vaccine applications (see below) the homology between a selected gene encoding a protein of the 37 kD protein family and SEQ ID NO: 1, 2 or 3 will preferably be somewhat higher than 60%. Although a 60% homology with SEQ ID NO: 1, 2 or 3 would be acceptable, a homology of > 70 % would be preferred. A homology of > 80% is more preferred, whereas an homology of > 90% is most preferred.

In exceptional cases, a member of the 37 kD protein may be found to have a nucleic acid homology that is below the level given above. This may e.g. be caused by an exceptionally large deletion. Nevertheless, such genes belong to the family of genes encoding a protein of the 37 kD protein family if they hybridise with any of the genes of which the nucleic acid sequence is given in SEQ ID NO: 1, 2 or 3 under stringent conditions. The conditions for hybridisation are given in Example 1.

[0006] Another way of determining if a protein belongs to the proteins of the 37 kD protein family is based upon binding of the protein to a specific antibody. This way of characterising the proteins of the 37 kD family is provided below. The characterisation is based upon the specific reaction of proteins of the 37 kD protein family with the monoclonal antibody F4.2F8. This monoclonal antibody binds to most proteins of the 37 kD family tested so far. The monoclonal antibody is highly specific; a common characteristic of all monoclonal antibodies. A hybridoma cell line producing the monoclonal antibody F4.2F8 has been deposited with the European Collection of Cell Cultures (ECACC), Centre for Applied Microbiology & Research (CAMR), Salisbury, Wiltshire SP4 0JG United Kingdom, under accession number 99031816. Therefore, all proteins binding to F4.2F8 are considered to be members of the 37 kD protein family. It can not be excluded that occasionally a strain is isolated having a protein of the 37 kD protein family that does not bind to monoclonal antibody F4.2F8. In such exceptional cases, the gene encoding the protein will however still hybridise to a gene of which the nucleic acid sequence is given in SEQ ID NO: 1, 2 or 3, or the gene will show a homology of at least 60 % with the nucleic acid sequence is given in SEQ ID NO: 1, 2 or 3.

[0007] In a first embodiment, the invention provides a nucleic acid sequence of *Babesia divergens* that encodes a protein of the 37 kD protein family or an immunogenic fragment thereof.

[0008] It could be shown by sequence analysis of the genes encoding the 37 kD protein family in the various strains, that the difference in size is often due to the presence, absence or polymorphism of small nucleic acid deletions in the gene. This is shown in Fig. 1. This figure shows a sequence comparison between the *B. divergens* strains Rouen 1987 (R), Weybridge 8843 (W) and Y5. It can be seen that strains W and Y5 encode a 35 kD variant form, whereas strain R encodes a 37 kD variant.

[0009] Next to the presence/absence of the deletion in W and Y5 compared to R, there are minor variations in the overall nucleic acid sequence of the gene encoding a protein of the 37 kD protein family in the respective *Babesia* strains. These variations may have no effect on the amino acid sequence of the polypeptide, in case that the modification is such that the variant triplet codes for the same amino acid. This is due to variation is based upon the phenomenon of degeneracy of the genetic code. It happens e.g. that due to natural mutation the G in the triplet CTC, coding for the amino acid Leucine, is replaced by a C, also coding for Leucine, or that the G in GAG coding for glutamic acid is replaced by an A, which triplet still encodes glutamic acid. Such a mutation is a silent mutation, i.e. it does not show at the amino acid level. Such silent modifications are very frequently found in nature, when comparing e.g. two different field isolates of *Babesia divergens*. This phenomenon holds for all amino acids, except Met and Trp. Thus, it is obvious, that the protein family of the present invention can not only be coded for by the nucleotide sequences given in SEQ ID NO: 1-3 but also by a very large variety of other sequences, all encoding the identical protein. It therefore goes without saying that any nucleic acid sequence encoding the proteins of SEQ ID NO: 1, 2 or 3 of the present invention as well

described, e.g. by Neurath et al. in "The Proteins" Academic Press New York (1979). Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia, Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Other amino acid substitutions include Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Thr/Phe, Ala/Pro, Lys/Arg, Leu/Ile, Leu/Val and Ala/Glu. Based on this information, Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science, 227, 1435-1441, 1985) and determining the functional similarity between homologous proteins. Such amino acid substitutions of the exemplary embodiments of this invention, as well as variations having deletions and/or insertions are within the scope of the invention as long as the resulting proteins retain their immunoreactivity. Thus, variations not essentially influencing the immunogenicity of the protein compared to the wild type protein as depicted in SEQ ID NO: 4-6 are considered to fall within the scope of the invention. Those variations in the amino acid sequence of a certain 37 kD protein according to the invention that still provide a protein capable of inducing protection against infection with *B. divergens* or at least against the clinical manifestations of the infection are considered as "not essentially influencing the immunogenicity".

[0011] In a preferred form, the invention relates to a nucleic acid sequence encoding a 37 kD protein or an immunogenic fragment thereof. Such a sequence could e.g. be isolated from an R strain, or any other strain encoding a protein of the 37 kD protein family with a molecular weight of 37 kD.

[0012] In a more preferred form of this embodiment the gene encoding the 37 kD protein has the sequence depicted in SEQ ID NO: 1.

[0013] In another preferred form, the invention relates to a nucleic acid sequence encoding a 35 kD protein or an immunogenic fragment thereof. This sequence could e.g. be isolated from a W strain, or any other strain encoding a protein of the 37 kD protein family with a molecular weight of 35 kD.

[0014] In a more preferred form of this embodiment the gene encoding the 35 kD protein has the sequence depicted in SEQ ID NO: 2 or 3.

[0015] The invention also relates to nucleic acid sequences encoding a protein of the 37 kD protein family that is capable of binding to the monoclonal antibody F42F8.

[0016] A definition of immunogenic fragments of a protein of the 37 kD protein family will be given below, where another embodiment of the invention, i.e. vaccination is discussed.

[0017] In general, the term "protein" refers to a molecular chain of amino acids with biological activity. A protein is not of a specific length and can, if required, be modified *in vivo* or *in vitro*, by, for example, glycosylation, amidation, carboxylation or phosphorylation; thus, inter alia, peptides, oligopeptides and polypeptides are included within the definition.

[0018] Since the present invention discloses the nucleic acid sequence encoding members of the 37 kD protein family, it is now for the first time possible to obtain such proteins in sufficient quantities. This can e.g. be done by using expression systems to express the gene encoding the protein of the 37 kD protein family.

An essential requirement for the expression of the nucleic acid sequence is an adequate promoter operably linked to the nucleic acid sequence. It is obvious to those skilled in the art that the choice of a promoter extends to any eukaryotic, prokaryotic or viral promoter capable of directing gene transcription in cells used as host cells for protein expression. Therefore, an even more preferred form of this embodiment relates to recombinant DNA molecules, i.e. DNA molecules according to the invention to which regulating sequences enabling expression of that nucleic acid sequence have been added. This can be obtained by means of e.g. standard molecular biology techniques. (Maniatis/Sambrook (Sambrook, J. Molecular cloning: a laboratory manual, 1989, ISBN 0-87969-309-6).

[0019] When the host cells are bacteria, useful expression control sequences which may be used include the Trp promoter and operator (Goeddel, et al., Nucl. Acids Res., 8, 4057, 1980), the lac promoter and operator (Chang, et al., Nature, 275, 615, 1978), the outer membrane protein promoter (Nakamura, K. and Inoué, M., EMBO J., 1, 771-775, 1982), the bacteriophage lambda promoters and operators (Bermat, L. et al., Nucl. Acids Res., 11, 4677-4688, 1983), the  $\alpha$ -amylase (*B. subtilis*) promoter and operator, termination sequences and other expression enhancement and control sequences compatible with the selected host cell.

When the host cell is yeast, useful expression control sequences include, e.g.  $\alpha$ -mating factor. For insect cells the polyhedrin or p10 promoters of baculoviruses can be used (Smith, G.F. et al., Mol. Cell. Biol. 3, 2156-65, 1983). When the host cell is of mammalian origin illustrative useful expression control sequences include the SV-40 promoter (Berman, P.W. et al., Science, 222, 524-527, 1983) or the metallothionein promoter (Brinster, R.L., Nature, 296, 39-42, 1982) or a heat shock promoter (Voellmy et al., Proc. Natl. Acad. Sci. USA, 82, 4949-53, 1985). Alternatively, expression control sequences present in *Babesia* may also be applied. For maximising gene expression, see also Roberts and Lauer (Methods in Enzymology 68, 479, 1979).

with Publication number 2 714 074, and in US NTIS Publication No US 08/043109 (Hoffman, S. and Rogers, W. Public Date 1 December 1993).

[0021] Another embodiment of the invention relates to Live Recombinant Carrier microorganisms (LRCs) comprising a gene encoding a protein of the 37 kD protein family according to the invention. Such micro-organisms are e.g. bacteria and viruses. These LRC micro-organisms are micro-organisms in which additional genetic information has been cloned. Animals infected with such LRCs will produce an immunogenic response not only against the immunogens of the vector, but also against the immunogenic parts of the polypeptide(s) for which the genetic code is additionally cloned into the LRC, e.g. the 37 kD protein.

As an example of bacterial LRCs, attenuated *Salmonella* strains known in the art can attractively be used.

[0022] Live recombinant carrier parasites have already been described by Vermeulen, A. N. (Int. Journ. Parasitol. 28: 1121-1130 (1998)).

Also, LRC viruses may be used as a way of transporting the nucleic acid sequence into a target cell. Live recombinant carrier viruses are also called vector viruses. The site of integration of the gene encoding a 37 kD protein may be a site in a viral gene that is not essential to the virus, or a site in an intergenic region. Viruses often used as vectors are Vaccinia viruses (Panicali et al, Proc. Natl. Acad. Sci. USA, 79: 4927 (1982), Herpesviruses (E. PA. 0473210A2), and Retroviruses (Valerio, D. et al; in Kaufman, S.J., Dicke, K.A., Lotzova, E. and Pluznik, D.H. (Eds.), Experimental Haematology today 1988, Springer Verlag, New York, pp. 92-99 (1989)).

[0023] The technique of *in vivo* homologous recombination, well-known in the art, can be used to introduce a recombinant nucleic acid sequence into the genome of a bacterium, parasite or virus of choice, capable of inducing expression of the inserted nucleic acid sequence according to the invention in the host animal.

[0024] Furthermore the invention relates to a host cell containing a nucleic acid sequence encoding the protein according to the invention, or a recombinant nucleic acid molecule encoding the protein under the control of regulating sequences enabling expression of the protein encoded by said nucleic acid sequence.

The invention also relates to a host cell containing a live recombinant carrier containing a nucleic acid molecule encoding a protein of the 37 kD family, or a recombinant nucleic acid molecule encoding a protein of the 37 kD family under the control of regulating sequences enabling expression of the protein encoded by said nucleic acid sequence. A host cell may be a cell of bacterial origin, e.g. *Escherichia coli*, *Bacillus subtilis* and *Lactobacillus* species, in combination with bacteria-based plasmids as pBR322, or bacterial expression vectors as pGEX, or with bacteriophages. The host cell may also be of eukaryotic origin, e.g. yeast-cells in combination with yeast-specific vector molecules, or higher eukaryotic cells like insect cells (Luckow et al, Bio-technology 6: 47-55 (1988)) in combination with vectors or recombinant baculoviruses, plant cells in combination with e.g. Ti-plasmid based vectors or plant viral vectors (Barton, K.A. et al; Cell 32: 1033 (1983), mammalian cells like Hela cells, Chinese Hamster Ovary cells (CHO) or Crandell Feline Kidney-cells, also with appropriate vectors or recombinant viruses.

[0025] Another objective of the invention is to provide an efficient vaccine against *Babesia divergens* infection, or at least against the clinical manifestations of the infection.

It was now surprisingly found, that an immunological response that provides immunity against infection with the *Babesia* parasite, or at least an immunological response that sufficiently reduces the level of clinical manifestations upon infection (as shown e.g. by a decrease of the haematocrit value), can be obtained by vaccination with vaccines comprising a protein of the 37 kD protein family or an immunogenic fragment thereof.

[0026] When a polypeptide is used for e.g. vaccination purposes or for raising antibodies, it is however not necessary to use the whole polypeptide. It is also possible to use a fragment of that polypeptide that is capable, as such or coupled to a carrier such as e.g. KLH, of inducing an immune response against that polypeptide, a so-called immunogenic fragment. An "immunogenic fragment" is understood to be a fragment of the full-length protein of the 37 kD family, that still has retained its capability to induce an immune response in the host, i.e. comprises a B- or T cell epitope. At the moment, a variety of techniques is available to easily identify DNA fragments encoding antigenic fragments (determinants). The method described by Geysen et al (Patent Application WO 84/03564, Patent Application WO 86/06487, US Patent NR. 4 833,092, Proc. Natl. Acad. Sci. 81: 3998-4002 (1984), J. Imm. Meth. 102: 259-274 (1987), the so-called PEPSCAN method is a easy to perform, quick and well-established method for the detection of epitopes, the immunologically important regions of the protein. The method is used worldwide and as such well-known to man skilled in the art. This (empirical) method is especially suitable for the detection of B cell epitopes. Also, given the sequence of the gene encoding any protein, computer algorithms are able to designate specific polypeptide fragments as the immunologically important epitopes on the basis of their sequential and/or structural agreement with epitopes that are now known. The determination of these regions is based on a combination of the hydrophilicity criteria according to Hopp

line 10: 3-7 (1992), Berzowsky for HIV epitopes, The FASEB Journal 5:2412-2418 (1991).

[0027] Therefore, another embodiment of the invention relates to vaccines inducing protection against infection with *B. divergens* or the clinical manifestations of the infection, comprising at least one protein of the 37 kD protein family or an immunogenic fragment thereof. It is clear that the protein of choice selected from the 37 kD protein family, or the gene encoding that protein can be a 35 kD protein or a 37 kD protein, or a mixture of the two. The protein or the gene encoding the protein can e.g. be chosen from *Babesia divergens* strain Rouen 1987 or Weybridge 8843 or any variants from other isolates.

[0028] More surprisingly it was also found, that proteins of the 37 kD protein family are capable of inducing antibodies that do not only protect against infection with a homologous *Babesia divergens* strain, but also with heterologous strains. This means i.e. that a vaccine based upon a 35 kD protein of e.g. *B. divergens* strain Weybridge induces protection against a 37 kD protein of e.g. *B. divergens* strain Rouen and *vice versa*.

Also, it was unexpectedly found that antibodies against a protein of the 37 kD protein family are capable of conferring *in vivo* immunity against both homologous and heterologous challenge.

This is the more surprising for the following reason: antibodies against the 17 kD protein mentioned above are abundantly found after experimental infection and after immunisation with fraction 4. Moreover,  $\alpha$ -Bd17, a monoclonal antibody against the 17 kD protein was described as the relevant vaccine component in passive vaccination against *Babesia* (Pridgout et al., Experimental Parasitology 77: 425-434 (1993), see above) on the basis of the fact that it dramatically inhibits *in vitro* growth of the parasite. On the basis thereof, the 17 kD protein was expected to be the relevant vaccine component. This antibody, however, when used as a reference in *in vivo* experiments, did unexpectedly not confer any protection at all, nor in a homologous nor in a heterologous challenge experiment (as will be seen in Example 4).

[0029] It was even more surprisingly found, that a vaccine comprising a mixture of two or more different proteins of the 37 kD family exhibit a synergistic effect compared to vaccines comprising the same amount of protein but from only one member of the 37 kD protein family. This has the advantage that vaccines comprising a mixture of two or more different proteins of the 37 kD protein family may comprise a lower overall amount of antigenic material compared to vaccines comprising a protein of only one member of the 37 kD protein family.

Thus in a preferred form, this embodiment of the invention relates to vaccines comprising two or more different proteins of the 37 kD protein family.

[0030] The following may serve as an example: a vaccine comprising a 35 kD protein and a 37 kD protein exhibits a synergistic effect compared to vaccines comprising the same amount of protein but from only 37 or 35 kD protein. A vaccine comprising both 50-75% of a 37 kD protein and 25-50 % of a 35 kD protein gives a level of protection against disease that is between 33% and 50% better than that found when the same amount of pure 37 kD protein or 35 kD protein was used as a vaccine. This holds true for challenge with strains comprising a 37kD protein as well as for strains comprising a 35 kD protein. This has the advantage that relatively low amounts of immunogenic material can be used, yet giving rise to a sufficiently high immune response.

Such vaccines can be made e.g. by admixing the desired amounts of the purified proteins, but also by admixing the desired amounts of cells comprising the different proteins. Therefore, in a more preferred form, this embodiment of the invention relates to vaccines comprising both 50-75% of a 37 kD protein of the 37 kD protein family and 25-50 % of a 35 kD protein of the 37 kD protein family.

[0031] Such vaccines can alternatively be made by admixing live recombinant carriers encoding the 37 kD protein and encoding the 35 kD protein according to the invention.

Alternatively, in the case of DNA-vaccines, mixtures of recombinant DNA molecules each capable of expressing one of the proteins can be made.

[0032] It is clear, that a vaccine for the protection of cattle against *Babesia divergens* infection or the clinical manifestations thereof can also be based upon the administration of antibodies against a protein of the 37 kD protein family. As a source of antibodies a polyclonal antiserum raised in e.g. rabbits against a member of the 37 kD protein family can be used. As described above, antisera against one protein of the 37 kD protein family show cross protection against other proteins of the 37 kD protein family. Therefore, any protein of the 37 kD protein family can be used for the induction of such a polyclonal antiserum. Also, neutralising monoclonal antibodies against a protein of the 37 kD protein family, such as e.g. the monoclonal antibody F4.2F8 can be used.

Thus, the invention also relates to vaccines protecting against infection of cattle with *B. divergens* or the clinical manifestations of the infection, that comprise antibodies against a protein of the 37 kD protein family.

[0033] Also, a vaccine according to the invention may comprise a recombinant DNA molecule comprising a nucleic acid sequence according to the invention and regulating sequences enabling expression of that nucleic acid sequence

the 37 kD protein family as carried by the LRC. As a result, the protein of the 37 kD protein family is expressed and presented to the immune system in a way that closely mimics the natural way of expression and immune presentation. Therefore, vaccination with a LRC carrying a gene of the 37 kD protein family or an immunogenic fragment thereof can advantageously be used.

Thus in another form of the embodiment relating to vaccines, the vaccine inducing protection against infection with *B. divergens* or at least against the clinical manifestations of the infection, comprises a live recombinant carrier according to the invention.

[0035] When a vaccine is made on the basis of a protein of the 37 kD protein family or an immunogenic fragment thereof obtained from an expression system, in principle the protein can be purified from the proteins of the host cells of the expression system. This is however not always necessary. It is very well possible to incorporate in a vaccine the cells that have been used for the expression of the protein of choice from the 37 kD protein family.

Thus, in still another form of the embodiment relating to vaccines, the vaccine inducing protection against infection with *B. divergens* or at least against the clinical manifestations of the infection, comprises a host cell according to the invention.

[0036] Additionally, the vaccine according to the invention may comprise one or more immunogenic proteins of other pathogens. This is advantageous, since vaccination with such a vaccine makes it possible to immunise an animal against two or more diseases in one vaccination step.

There are several ways of obtaining such a vaccine. One is admixing a protein according to the invention with one or more immunogenic proteins of other pathogens. Another possibility is the cloning of a heterologous DNA sequence encoding an immunogenic protein of another pathogen into a live recombinant carrier according to the invention.

[0037] Preferably the immunogenic protein is chosen from the group of cattle pathogens, consisting of Bovine Herpesvirus, bovine Viral Diarrhoea virus, Parainfluenza type 3 virus, Bovine Paramyxovirus, Foot and Mouth Disease virus, *Pasteurella haemolytica*, Bovine Respiratory Syncytial Virus, *Theileria parva*, *Theileria annulata*, *Babesia bovis*, *Babesia bigemina*, *Babesia major*, *Trypanosoma species*, *Anaplasma marginale*, *Anaplasma centrale* or *Neospora caninum*.

[0038] Furthermore, the heterologous DNA sequence may encode a cytokine such as interleukins, TNF and interferons. Several cytokines, e.g. interferons are known to play an important role as immune modulators. Thus it may be advantageous to include genetic information for this kind of molecule into said section.

[0039] It is obvious that a heterologous DNA sequence can be introduced at a certain site in said section, e.g. in a restriction site without deleting any nucleotides from the section. On the other hand, it is possible to exchange one or more nucleotides with heterologous DNA sequences of equal or different length.

[0040] Vaccines according to the present invention can be made e.g. by merely admixing of a protein of the 37 kD protein family or an immunogenic fragment thereof, either as such or coupled to a suitable carrier molecule, and a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier is understood to be a compound that does not adversely affect the health of the animal to be vaccinated, at least not to the extent that the adverse effect is worse than the effects seen when the animal is not vaccinated. A pharmaceutically acceptable carrier can be e.g. sterile water or a sterile physiological salt solution. In a more complex form the carrier can e.g. be a buffer.

Also, the vaccine can be made by admixing a nucleic acid sequence encoding a protein of the 37 kD family, a recombinant DNA molecule encoding a protein of the 37 kD family, a live recombinant carrier or a host cell according to the invention and a pharmaceutically acceptable carrier.

[0041] Thus another embodiment of the invention relates to methods for the preparation of a vaccine comprising the admixing of a protein of the 37 kD protein family or an immunogenic fragment thereof, antibodies against a protein of the 37 kD family, a nucleic acid sequence according to the invention, a recombinant DNA molecule according to the invention, a live recombinant carrier according to the invention or a host cell according to the invention and a pharmaceutically acceptable carrier.

[0042] The vaccine according to the present invention may in a preferred presentation also contain an adjuvant. Adjuvants in general comprise substances that boost the immune response of the host. A number of different adjuvants are known in the art. Examples of adjuvants are Freund's Complete and Incomplete adjuvant, vitamin E, non-ionic block polymers and polyamines such as dextran sulphate, cartopol and pyran. Also very suitable are surface active substances such as Quil A<sup>(1)</sup>.

[0043] Saponins are a preferred adjuvant. Saponins are preferably added to the vaccine at a level between 20 and 150 µg/ml. Within the group of saponins, the saponin Quil A is a more preferred adjuvant. Furthermore, peptides such as muramyl peptides, dimethylglycine, tuftsin, are often used. Next to these adjuvants,

the vaccine according to the invention may also contain one or more additional immunogenic fragments of the protein of



242 380)

Often the vaccine is mixed with stabilisers, e.g. to protect degradation-prone polypeptides from being degraded, to enhance the shelf life of the vaccine, or to improve freeze-drying efficiency. Useful stabilisers are i.a. SPGA (Bovarnik et al., J. Bacteriology 59: 509 (1956)), skimmed milk, gelatin, bovine serum albumin, carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers such as alkaline metal phosphates.

Freeze-drying is an efficient method for conservation. Freeze-dried material can be stored and kept viable for many years. Storage temperatures for freeze-dried material may well be above zero degrees, without being detrimental to the material.

Freeze-drying can be done according to all well-known standard freeze-drying procedures.

Therefore, in a preferred embodiment, the vaccine is in a freeze-dried form.

In addition, the vaccine may be suspended in a physiologically acceptable diluent. Such a diluent can e.g. be as simple as sterile water, or a physiological salt solution.

It goes without saying, that other ways of adjuvating, adding vehicle compounds or diluents, emulsifying or stabilising a polypeptide are also embodied in the present invention.

[0044] The vaccine according to the invention can be administered in a conventional active immunisation scheme: single or repeated administration in a manner compatible with the dosage formulation, and in such amount as will be prophylactically effective, i.e. the amount of immunising antigen or recombinant micro-organism capable of expressing said antigen that will induce immunity in cattle against challenge by virulent *Babesia divergens* parasites. Immunity is defined as the induction of a significant level of protection in a population of cattle after vaccination compared to an unvaccinated group.

[0045] For live viral vector vaccines the dose rate per animal may range from  $10^3$  to  $10^8$  pfu (but even  $< 1000$  pfu might be sufficient e.g. for Bovine Herpesvirus carrier virus). A typical subunit vaccine according to the invention comprises 0.1 to 100 µg of the polypeptide (or variant or fragment thereof) according to the invention. Preferably at least 5 µg will be present. Such vaccines can be administered e.g. intradermally, subcutaneously, intramuscularly, intraperitoneally, intravenously, orally or intranasally.

[0046] An alternative and efficient way of vaccination is direct vaccination with DNA encoding the relevant antigen. Direct vaccination with DNA encoding proteins has been successful for many different proteins. (As reviewed in e.g. Donnelly et al., The Immunologist 2: 20-26 (1993)). In the field of anti-parasite vaccines, protection against e.g. *Plasmodium yoelii* has been obtained with DNA-vaccination with the *Plasmodium yoelii* circumsporozoite gene (Vaccine 12: 1529-1533 (1994)). Protection against *Leishmania major* has been obtained with DNA-vaccination with the *Leishmania major* surface glycoprotein gp63 gene (Vaccine 12: 1534-1536 (1994)).

This way of vaccination is also very attractive for the vaccination of cattle against Babesiosis. Therefore, the invention also relates to vaccines comprising DNA encoding one or more proteins of the 37 kD protein family or an immunogenic fragment thereof.

[0047] Since infection with *Babesia divergens* may progress relatively slowly, it may take some time before the diagnosis of *Babesia divergens* infection is made. In many cases in diseased animals *Babesia divergens* infection is only found to be the cause of the disease after the occurrence of haemoglobinuria (visible since it turns the urine red), which means that the disease has already significantly progressed.

Moreover, babesiosis infection is found in different forms. One form is characterised by the fact that *Babesia* antigens are found in the plasma, but no antibodies have yet been formed. This situation is found in acutely infected animals.

[0048] A second form is the form in which the *Babesia* parasites have been cured and the plasma does contain antibodies against *Babesia* antigens but no *Babesia* antigens. This is the situation found in animals that have developed a sterile immunity against *Babesia*. A third form is the form in which both antigens and antibodies are found in the plasma. Animals having this form of infection are immune against *Babesia* but they are at the same time carrier of the parasite. These animals are a potential source of infection for other animals.

Thus in order to determine the *Babesia* infection status of an animal it is important to determine both the *Babesia* antigen levels and the anti-*Babesia* antibody level in the serum. Therefore diagnostic tools preferably capable of differentiating between the various forms are highly wanted.

Another embodiment of this invention provides such diagnostic tools.

[0049] A diagnostic test for the detection of *Babesia divergens* antibodies in sera can be e.g. a simple ELISA-test in which purified protein of the 37 kD protein family or an antigenic fragment thereof is coated to the wall of the wells of an ELISA plate. Incubation with serum from mammals to be tested, followed by e.g. incubation with a labelled antibody

[0050]



*Babesia divergens* in which the method comprises the incubation of serum with protein of the 37 kD protein family or an antigenic fragment thereof.

[0051] A diagnostic test for the *Babesia* antigens and therefore suitable for the detection of *Babesia divergens* parasites can e.g. also be a standard ELISA test in which the walls of the wells of an ELISA plate are coated with antibodies directed against a protein of the 37 kD protein family.

[0052] The invention also relates to methods for the detection of *Babesia divergens* parasites, that comprise the incubation of serum with antibodies against a protein of the 37 kD protein family.

[0053] A quantitative, or competition ELISA not only indicating the presence or absence of *Babesia* antigens but also indicating the amount of *Babesia* antigens, is also part of the invention. Such an ELISA is well-known in the art.

Thus this embodiment of the invention also relates to competitive ELISAs.

[0054] Antibodies or derivatives thereof (e.g. fragments such as Fab, F(ab')<sub>2</sub> or Fv fragments), which are directed against proteins of the 37 kD protein family according to the invention and have potential uses in passive immunotherapy, diagnostic immunoassays and in the generation of anti-idiotypic antibodies can be made according to standard techniques as indicated below.

The proteins or immunogenic fragments thereof according to the invention as characterised above can be used to produce antibodies, which may be polyclonal, monospecific or monoclonal (or derivatives thereof). If polyclonal antibodies are desired, techniques for producing and processing polyclonal sera are known in the art (e.g. Mayer and Walter, eds. *Immunochemical Methods in Cell and Molecular Biology*, Academic Press, London, 1987).

Monoclonal antibodies, reactive against the protein according to the invention (or variants or fragments thereof) according to the present invention, can be prepared by immunising inbred mice by techniques known in the art (Kohler and Milstein, *Nature*, 256, 495-497, 1975).

Antibodies against any of the polypeptides of the present invention and made e.g. in one of the manners described above, can be used i.a. for vaccination purposes, especially in immunocompromised animals.

[0055] All kinds of antibodies against a protein of the 37 kDa protein family can be used in the diagnostic tests described above. Polyclonal antibodies obtained by injection of the Bd37 protein to an animal have a high affinity with all proteins of the 37 kDa protein family. Nevertheless, the antibodies of preference are specific antibodies made by the hybridoma cell line deposited with the ECACC under accession number 99031816. They have a high affinity with all proteins of the 37 kDa protein family tested so far.

[0056] The diagnostic tests for the detection of antibodies against *Babesia divergens* are preferably in the form of a kit, comprising 37 kD protein in a purified form. The antigens could e.g. be purified through standard protein separation techniques over a suitable column. Another possibility is separation on a PAAGE gel followed by Western-blotting. On the Western-blot, the 37 kD protein will form a specific band, separated from other *Babesia divergens* protein bands, and thus is also considered to be purified. Also, a pure form of the protein can be obtained by expressing one of the nucleic acid sequences according to the invention.

In principle, the easiest way of making such a diagnostic test system is to use purified whole 37 kD protein as explained above. It is however very well possible to use only part of the 37 kD protein. This with the proviso that the fragment used still comprises an antigenic determinant of the protein. All antigenic determinants of the 37 kD protein will induce antibodies by definition. Therefore the use of a 37 kD protein fragment comprising even one single antigenic determinant of the 37 kD protein will be capable of binding to anti-37 kD protein antibodies.

Therefore, in another embodiment the present invention relates to a diagnostic kit for the detection of antibodies against *Babesia divergens*, in which the kit comprises a purified protein of the 37 kD protein family or fragments thereof still comprising an antigenic determinant.

As mentioned above, the protein can be purified directly from cells infected with the parasite. It is however also possible to use standard expression systems such as bacterial, parasite, yeast, baculovirus or mammalian expression systems, of which a large variety has been described in the art, for the expression of the 37 kD protein. The protein thus expressed can easily be purified.

[0057] In still another embodiment the present invention relates to a diagnostic kit for the detection of *Babesia divergens* parasites, in which the kit comprises antibodies reactive with an antigenic determinant on a protein of the 37 kD family.

[0058] Finally, still another embodiment of the invention relates to the use of a protein of the 37 kD protein family or a mixture of such proteins for the manufacture of a vaccine inducing protection against infection of cattle with *B. divergens* or the clinical manifestations of the infection. Such manufacture is described above where methods for the preparation of vaccines are described.

## EXAMPLES

## Example 1

Growth of *Babesia divergens* and Genomic DNA extraction

[0059] An *in vitro* culture of *Babesia divergens* was grown according to Grande *et al.* (Parasitology, 115, 81-84 (1997)).

When parasitemia from *in vitro* culture reached 50 %, *B. divergens* parasitized erythrocytes were pelleted by centrifugation 5 min. at 2500 g.

Supernatant was centrifuged 20 min. at 15000 g; the pellet, containing merozoites, was washed twice in RPMI.

Infected red blood cells were lysed in 5 volumes of lysis buffer (5 mM EDTA, 100 mM NaCl, 20 mM Tris pH 7.5, 0.5 % Triton X-100, 0.5 % SDS) supplemented with proteinase K at a final concentration of 1 mg/ml, 1 h at 37°C.

DNA extraction was performed in 3 steps with phenol, phenol-chloroform, and finally chloroform : isoamyl alcohol (24 : 1); addition of 1 volume of phenol or phenol-chloroform or chloroform : isoamyl alcohol (24 : 1), vortex 1 min., centrifugation 5 min. at 10000 g, and transfer of the top aqueous phase in a fresh tube.

DNA was then precipitated by 2 volumes of cold ethanol 100 % and 1/10 volume of 3M sodium acetate pH 5.6, 1 h at 80°C. After centrifugation 15 min. at 10000 g at 4°C, DNA pellet was washed with ethanol 70 %, dried and resuspended in sterile water.

## Plasmid miniprep

[0060] A single plasmid colony was picked up into a tube containing 1.5 ml of LB medium (10 g of bactotryptone, 5 g of yeast extract and 5 g of NaCl in 1 litre of distilled water, mixed and autoclaved), containing the appropriate antibiotic and incubated overnight at 37°C. Cells, pelleted by centrifugation 30 seconds at 10000 g, were firstly dissolved in 200 µl of a solution containing 100 mM Tris-HCl pH 7.5; 10 mM EDTA, secondly in 200 µl of a solution containing 200 mM NaOH, 1 % SDS and thirdly, after 5 min. at room temperature, in 200 µl of a solution containing 3 M potassium acetate and 5 M acetic acid. After incubation during 5 min. at 4°C and centrifugation 5 min. at 10000 g, plasmid DNA contained in the supernatant was precipitated by 0.7 volumes of isopropanol; it was then dissolved in 200 µl of TE buffer (10 mM Tris-HCl pH 7.5; 1 mM EDTA). For sequencing, a phenol-chloroform extraction and an ethanol precipitation were performed, as previously described.

## cDNA library construction

[0061] A cDNA library was obtained from mRNA derived from *B. divergens* Rouen 1987 cultures as follows: 10<sup>9</sup> infected erythrocytes were pelleted by centrifugation of culture and treated by a lytic agent containing guanidine thiocyanate, using RNA agents<sup>®</sup> Total RNA Isolation System kit (Promega). Total RNA were then purified by phenol : chloroform extraction and precipitated with isopropanol. With total RNA as the starting material, the poly(A) mRNA fraction was isolated by hybridisation to a biotinylated oligo(dT) primer, using PolyA Tract mRNA Isolation Systems kit (Promega). The hybrids were captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand. mRNA were eluted by water and cDNA were synthesised, using ZAP Express<sup>™</sup> cDNA Synthesis kit (Stratagene).

cDNA synthesis was performed by reverse transcription, using a primer which contains a poly(dT) sequence and a *Xho*I restriction site. *Eco*R<sup>+</sup> adapters were added to the 5' end of 0.4-4 kbp cDNA fragments (size fractionated on Sephacryl S-500 spin Columns); after *Xho*I digestion, cDNA was ligated into the  $\lambda$  ZAP II Express Vector in a sense orientation (*Eco*R<sup>+</sup> *Xho*I) with respect to the lacZ promoter, using a ratio of 150 ng of cDNA to 1 µg of vector. Recombinant lambda phages were then packaged in G-japack II packaging extracts from Stratagene, and plated on NZY agar plates, using XL1-Blue MRF<sup>+</sup> cells, to be amplified and titrated as recommended by the manufacturer (Stratagene).

## cDNA library screening

[0062] The polyclonal antibody directed against the 37 kDa protein family ( $\alpha$  Bd37) was used for the screening of the cDNA library of *B. divergens* Rouen 1987.

For screening, 4000 recombinant phages of the cDNA library were plated using XL1-Blue MRF<sup>+</sup> cells. After lysis, cells were

[0063] Positive plates were transferred in 500 µl of SM buffer, incubated 1 h at 37°C and stored at 4°C. Following purification of these positive plates, cDNA encoding the 37 kDa protein was excised out of the λ-ZAPII phage in the form of the kanamycin resistant pBluescript MV phagemid vector, using a helper phage, as recommended by Stratagene.

#### cDNA sequencing

[0064] 1 µg of plasmids carrying cDNA encoding the 37 kDa protein of *B. divergens* Rouen were sequenced by using the <sup>32</sup>P sequencing kit (Pharmacia Biotech). After denaturation with NaOH, neutralisation and ethanol precipitation, cDNA was pelleted by centrifugation and resuspended in 10 µl of water. Annealing reactions were made using 10 pmol of primer and then, DNA were sequenced using the dideoxy method of Sanger *et al* ((1977), *Proc. Natl. Acad. Sci. USA* 74 (12) : 5463-7) using the reagents and procedures of Pharmacia Biotech. Primers useful for reading the entire sequence were synthesised by Eurogentec.

#### Analysis of the cDNA sequence encoding the 37 kDa protein

[0065] The sequence of the cDNA encoding the 37 kDa protein (1208 bp between restriction sites *EcoRI* and *XhoI*) showed the presence of a poly(A)<sub>18</sub> tail and a coding region of 1023 desoxyribonucleotides, giving a single open reading frame of 341 amino acids, without repetitive motif.

Analysis of the hydrophilicity profile revealed two hydrophobic sequences, at each extremity of the protein : from amino acids 1 to 18 at the N-terminal end and from amino acids 321 to 341 at the C-terminal end of the protein. The hydrophobic segment at the N-terminal end could correspond to the signal sequence of the 37 kDa protein and the presence of such a cleavable N-terminal signal sequence suggested that the 37 kDa protein is a secreted protein. However, the presence of another hydrophobic segment at the C-terminal end of the molecule indicated that the 37 kDa protein could interact or be anchored in the membrane of parasitic vesicles during the secretory pathway to be on the surface of the parasite at the end of this process. These observations are consistent with the pattern of immunofluorescence obtained on *B. divergens* infected erythrocyte since the labelling was observed both inside parasitic vesicles and on the surface of the parasite. The finding of the 37 kDa protein in the supernatant of *in vitro* culture could result in a cleavage of the molecule either from the surface of the parasite or from secreting vesicles.

#### Cloning of the 37 kDa protein gDNA from different isolates of *B. divergens* in pGEM®-T Vector

[0066] Genomic DNA (gDNA) of the *B. divergens* Rouen 1987, Weybridge 8843 and Y5 isolates were amplified by Polymerase Chain Reaction (PCR) and cloned in pGEM®-T vector System II (Promega), in order to be sequenced as described above.

gDNA from *B. divergens* Rouen 1987 and Weybridge 8843 were amplified using primer Bd37-5 (sense oligonucleotide), 5'-GAATTCACGACCAACGAATA-3', and Bd37-Low (antisense oligonucleotide), 5'-ACAGGATCCAAAAGCATACATAGCTGTCCACT-3', while gDNA from *B. divergens* Y5 were amplified using primer Bd37-Upp (sense oligonucleotide), 5'-CAAGGATCCTC:TAAGTACGATGAAAACCGAGTAA-3', and Bd37-Low. To perform the PCR, 100 ng of each gDNA were incubated with 0.5 µl of each primer at 10 µM, 4 µl of dNTP (1.25 mM each), 2.5 µl of 10X enzyme buffer and 1 unit of *Taq* DNA polymerase (Ozyme) in a final volume of 25 µl. The following conditions were used : 3 min. at 94°C, 30 cycles of 1 min. at 94°C, 1 min. at 50°C and 2 min. at 72°C, and then 5 min. at 72°C.

PCR products were purified by electroelution : they were subjected to a 1 % agarose gel electrophoresis. The expected band was visualised under UV, excised and subjected to electroelution in 400 µl of TAE buffer at 100 mA during 1 h. The electroeluted materials were phenol extracted and ethanol precipitated as described above.

The ligation reaction was performed by adding 50 ng of each gDNA to 50 ng of pGEM vector and 3 units of *T4* DNA ligase in 1 µl of the 10X appropriate buffer, during 5 h at 16°C. 2 µl of each ligation mix were used for the transformation reaction in JM109 High Efficiency Competent Cells, as recommended by Promega.

Recombinant vectors were visualised, using blue-white screening : some white colonies were picked up and transferred in 50 µl of sterile water. After vortex, tubes were boiled for 5 min. and centrifuged 1 min. at 10000 g : 10 µl of the supernatant were used for a PCR reaction as described above, with T3 and T7 primers, in order to verify the presence of the insert. For sequencing of the insert, a culture of 1.5 ml in LB of the recombinant vector was used for a plasmid miniprep, as previously described.

Analysis of the cDNA sequence encoding the 37 kDa protein family by hybridisation

Translation kit (Boehringer Mannheim), with the Redivue™ [ $\alpha$ - $^{32}$ P]dCTP (3000 Ci/mmol) at a final concentration of 20  $\mu$ Ci (Amersham) and then purified using Probe Purification after labelling kit (Jenrick) as recommended by manufacturer. Membranes are firstly incubated in a prehybridization solution (5xSSC, 0.5 % SDS and 1 g/l of Ficoll type 400, Polyvinylpyrrolidone and Bovine Serum Albumine), 1 h at 65°C and then hybridized overnight at 65°C in 10 ml of the same solution, supplemented with 1 mg of herring sperm DNA and the denaturated labelled probe. Membranes are washed three times for 15 min. at 65°C in 6xSSC and exposed to Biomax MR-1 film (PoliLabo) at -80°C, during 48 hours.

DNA that hybridises to the DNA on the hybrid-N membrane comprises a gene encoding a protein of the 37 kD protein family.

## Example 2

### Cloning of the Bd37 cDNA in the pGEX-A vector

[0068] In order to express a recombinant Bd37 protein, the cDNA was sub-cloned in the *E. coli* vector pGEX-A. In a first step, the entire coding region of the cDNA was amplified by PCR using primers Bd37 Upp (sense oligonucleotide) and Bd37 Low (antisense oligonucleotide), which contain *Bam*HI restriction sites. The PCR products was then sub-cloned in the pGEM<sup>®</sup>-T vector, as described above. In a second step, 500ng of pGEX-A vector and 500ng of the pGEM<sup>®</sup>-T vector containing Bd37 cDNA were digested with 30 units of *Bam*HI restriction enzyme, 3 h at 37°C, in the appropriate buffer (Life technologies), in order to be ligated.

After phenol/chloroforme extraction and ethanol precipitation, the pGEX-A vector was dephosphorylated, using 0.0015 units of Calf Intestinal Alkaline Phosphatase (Promega), 1 h at 37°C in the appropriate buffer and reprecipitated. The excised cDNA fragment was purified by electroelution, as described above.

The ligation of 100 ng of *Bam*HI-cut phosphatase treated pGEX-A vector with 100 ng of Bd37 cDNA fragment was performed using 15 units of T4 DNA ligase, in the appropriate buffer, during 1 h at 25°C. 1  $\mu$ l of the ligation mix were used to transform 75  $\mu$ l of Epicurian Coli<sup>®</sup> SURE<sup>®</sup>2 supercompetent cells, as recommended by the manufacturer (Stratagene).

Recombinants were determined by hybridisation: Hybond-N (Amersham) filters were applied 30 seconds on plates, denaturated 1 min. with 1.5M NaCl, 0.5N NaOH and neutralised with 1.5 M NaCl, 0.5 M Tris-HCl pH 7.5. The filters were rinsed in 2xSSC, air dried and exposed, DNA side up, to UV ( $\lambda$ >254nm) during 3 min.

A PCR was performed on *B. divergens* Rouen 1987 cDNA, using internal primers Bd37-E1 (sense oligonucleotide), 5'-CAAGGTGGTGC GAATTC AAAG 3' and Bd37-E4 (antisense oligonucleotide), 5'-ATACAATGATACCGAATTC AATGG-3'. 0.2  $\mu$ g of the electroeluted PCR fragment were used to synthesise a nucleotide probe using Nick Translation kit (Boehringer Mannheim). The probe was labelled with the Redivue™ [ $\alpha$ - $^{32}$ P]dCTP (3000 Ci/mmol) at a final concentration of 20  $\mu$ Ci (Amersham). The excess of triphosphate deoxyribonucleosides were discarded on a column of glass beads (Glasperlen 0.25-0.3 $\mu$ m from Braur) and Sephadex<sup>®</sup> G50 superfine (Pharmacia).

Membranes were prehybridised in 6xSSC + 2.5% non fat milk, 1 h at 65°C, and hybridized overnight at 65°C in 6xSSC + 2.5% non fat milk containing herring sperm DNA at 100  $\mu$ g/ml and the denaturated labelled probe. Membranes were washed three times for 15 min. at 65°C in 6xSSC and exposed to Biomax MR-1 film (PoliLabo) at -80°C, during two hours.

The correct orientation of the Bd37 fragment in the positive recombinant pGEX-A vector was controlled by performing PCR on the purified recombinant vector using the 5'pGEX primer (Pharmacia), combined with the internal Bd37-E4 primer.

[0069] The same procedure has been followed, using 100 ng of genomic DNA from *B. divergens* Weybridge 8843 as the starting material, in order to clone a gene encoding a 35 kDa protein in the pGEX-A vector and to express a recombinant Bd35 protein.

[0070] Lastly, as a control, 100 ng of native pGEX-A vector were used to transform 75  $\mu$ l of Epicurian Coli<sup>®</sup> SURE<sup>®</sup>2, supercompetent cells, in order to produce native glutathione S transferase (GST).

### Production of recombinant GST, GST-Bd37 and GST-6d35 proteins for a vaccination assay.

[0071] The different constructs which express GST or a fusion protein of Bd37 or Bd35 protein with GST, were induced by IPTG. The bacterial cultures were grown overnight and diluted 1:10 with LB medium supplemented with 10  $\mu$ M IPTG during 9 h. The

## Production of a recombinant 37 kDa protein in insect cells for a vaccination assay

[0072] For the production of the recombinant DNA in baculovirus, the cDNA encoding the 37 kDa protein was cloned according to standard procedures into the Bac-to-Bac™ vector provided by Life Technologies, 8717 Grovemont Circle, P.O. box 6009, Gaithersburg, MD 20884-9980, U.S.A.

Six T75 flasks were used for the culture of infected and non-infected SF9 cells, containing each  $7.5 \times 10^6$  SF9 cells in a final volume of 15 ml of HYQ-CCM3 medium (Hyclone Europe NV, Belgium). Three of these flasks were infected with 52.5 µl of Bd37-1 virus ( $9 \times 10^7$  pfu/ml), which corresponds to an infection of SF9 cells at a multiplicity of infection of 0.5. Infected and non-infected cells (RBC) were collected 60 h post-infection in a final volume of 7.5 ml of CCM3 medium and treated by 0.3% Triton X-100, 1 h at room temperature. An aliquot of 100 µl of infected and non-infected cells were dissociated in 10 µl of 10X Sample Buffer and boiled during 5 min. Two aliquots of 25 µl from each sample (one used for a Coomassie Blue staining and one used for an immunoblotting using the monoclonal antibody F4-2F8 at a dilution of 1:50) were submitted to a 12% SDS-PAGE. These infected cells, expressing the 37 kDa protein, and non-infected cells have been used to immunise gerbils, in order to test the immunoprotective effect of the 37 kDa protein.

### Example 3

#### Vaccination assay with a recombinant 35 and 37 kD protein of the 37 kDa protein family

[0073] The vaccination assay has been conducted on 5 groups of 10 gerbils, in order to test the immunoprotective effect of the recombinant Bd37-1 protein, either expressed in the procaryotic system (using the fusion proteins GST-Bd37 or GST-bd35) or in the eukaryotic Bac-to-bac system (using the recombinant Bac-Bd37). Three different doses of recombinant proteins, comprising of 5 µg, 25 µg and 125 µg, have been tested (See table 1). Negative controls groups have only received the equivalent of native GST or non-infected insect cells. Each vaccine preparation have been adjuvanted with the Quil-A adjuvant. A positive control group have received 300 µl of supernatant from *B. divergens* Rouen 1987 *in vitro* culture. A challenge control group only received  $10^6$  parasitized erythrocytes.

#### Animals

[0074] Gerbils (7-8 weeks of age) were used. The animals received food and water ad libitum.

#### Parasites

[0075] The challenge strain used was *B. divergens* Rouen 1987 and each animal received  $10^6$  parasitized erythrocytes in a final volume of 100 µl.

#### Vaccine preparation

[0076] Recombinant proteins (GST, GST-Bd35 or GST-Bd37 protein, infected or non-infected insect cells lysate) were produced as described above (cf. fig. 3).

Supernatant from *B. divergens* Rouen 1987 *in vitro* culture was harvested when parasitemia reached 30-40% in RPMI-Albumax, centrifuged at 1000 g for 15 min., passed through a filter-membrane (0.22 µm pore-size; Gelman Sciences) and stored at -80°C until use.

[0077] Each preparation (GST, GST-Bd35 or GST-Bd37 protein, infected or non-infected insect cells lysate and supernatant from *in vitro* culture) was diluted in RPMI in order to obtain 12 doses each containing the expected quantity of recombinant protein (or equivalent) in a final volume of 3.4 ml. 100 µl of a stock solution Quil A at 2.8 mg/ml have been added to each vaccine preparation. A dose of 300 µl has been injected subcutaneously to each gerbil.

Table 1

Group	Vaccine	Quantity	Nb of injection	Challenge
1	GST-Bd37	5 µg	12	R
2	GST-Bd35	5 µg	12	R
3	GST	5 µg	12	R
4	Supernatant	300 µl	12	R
5	Non-infected cells	300 µl	12	R
6	Non-infected cells	300 µl	12	C

Table 1 (continued)

Group	Vaccine	Quantity	Nb of injection	Challenge
5	GST	2.5µg	2	R
6	GST	12.5µg	2	R
7	GST	62.5µg	2	R
8	bac-Bd37	5µg	2	R
9	bac-Bd37	25µg	2	R
10	bac-Bd37	125µg	2	R
11	NIC	5µg	2	R
12	NIC	25µg	2	R
13	NIC	125µg	2	R
14	SN R	300µl	2	R
15	/	/	2	R
R : <i>B. divergens</i> Rouen 1987 strain NIC : non-infected insect cells SN R : supernatant from <i>B. divergens</i> Rouen 1987 <i>in vitro</i> culture				

### Vaccination procedure

[0078] Priming and booster infections have been done subcutaneously with a 3 weeks interval. Animals were challenged with *B. divergens* Rouen 1987 intraperitoneally, 3 weeks after the booster injection, with  $10^6$  parasitized erythrocytes (100 µl)/gerbil.

[0079] At days D-43, D-22 and D-1, a blood sample has been collected from the animals of each group. The serum samples of each group were tested by immunoprecipitation and immunofluorescence in order to analyse the humoral response of the gerbils against the recombinant 37 kDa protein.

### Results

#### Immunoprecipitation

[0080] The sera from each gerbil, from D-43, D-22 and D-1, were used to immunoprecipitate  $^{35}$ S-radiolabelled *B. divergens* Rouen 1987 total antigens.

Firstly, from figure 4, it can be seen that non-infected insect cells and native GST are not immunogenic in the sense that the humoral response doesn't cross-react with *B. divergens* antigens (groups 5 to 7 and 11 to 13) - the pattern observed corresponds to the non-specific response.

For all the sera from gerbils vaccinated with the 37 kDa recombinant protein, a strong humoral response against the protein is specifically observed (groups 1 to 4 and 8 to 10), whereas controls vaccinated with NIC (non-infected cells) or native GST are negative.

## Protection

[0081]

Table 2A :

Groups	1	2	3	4	5	6	7
Challenge doses	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites
Challenge strain	R	R	R	R	R	R	R
Number of gerbils	9	10	10	10	10	10	10
Vaccine	GST-37	GST-37	GST-37	GST-35	GST	GST	GST
Dose ( $\mu$ g)	5	25	125	25	2,5	12,5	62,5
Number of gerbils	9	10	10	10	0	0	0

Table 2B :

Groups	8	9	10	11	12	13	14	15
Challenge doses	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites	$10^6$ parasites
Challenge strain	R	R	R	R	R	R	R	R
Number of gerbils	9	10	10	10	10	10	10	10
Vaccine	Bac-Bd37	Bac-Bd37	Bac-Bd37	NIC	NIC	NIC	SN R	/
Dose ( $\mu$ g)	5	25	125	5	25	125	300 $\mu$ l	/
Number of gerbils	8	10	8	1	1	0	10	0

Table 2 : Protection with recombinant 37 kDa protein expressed in a procaryotic (A) or eucaryotic (B) system

## Conclusion

[0082] As can be seen from table 2A, a total immunoprotection is conferred to gerbils vaccinated with GST-Bd37 and GST-Bd35, respectively against a homologous or heterologous challenge with *B. divergens* Rouen 1987 (which encodes a 37kDa protein). Gerbils did not present any fall of haematocrit and any positive parasitemia. These results were obtained with very low (5  $\mu$ g) doses as well as with high doses (125  $\mu$ g) of recombinant protein. Contrary to this, all control gerbils, vaccinated with native GST, died.



system used

#### Example 4

#### Passive immunisation with monoclonal antibodies F4.2F8 and homologous or heterologous challenge

[0083] Hybridoma producing the mAb F4.2F8 were grown and the monoclonal antibodies were purified according to standard procedures for the preparation of monoclonal antibodies. The monoclonal antibodies were injected intraperitoneally in gerbils and the concentrations of F4.2F8 were maintained by multiple injections, starting one day before the challenge and continuing several days after.

As an antibody concentration of  $10^3$   $\mu$ g/ml is needed (1 % of total gerbil immunoglobulins), 0.5 mg of antibodies in culture medium was daily injected per gerbil. The experiment was continued during 7 days after challenge.

[0084] Two different challenge strains were used: *B. divergens* Rouen 1987 (comprising the 37 kDa protein) and Weybridge 8843 (comprising the 35 kDa protein). Gerbils were challenged by a high dose ( $10^6$  parasitized erythrocytes by gerbil) or a low dose ( $10^3$  parasitized erythrocytes by gerbil). The volume of the challenge dose ( $10^6$  PE or  $10^3$  PE) was 100  $\mu$ l.

For each strain of challenge, a first control group (isotype control) has received an unrelated mouse monoclonal antibody IgG2a, which is an antibody against a non 37 kDa protein having however the same isotype as F4.2F8, in order to test the non specific activity. A second control group (antibody control) has received the monoclonal antibody  $\alpha$ -Bd17, directed against another *B. divergens* protein, the 17 kDa protein described above (Précigout *et al.* (1993), *Exp. Parasitol.* 77 (4): 425-34). A last control has not received any injection of antibodies (no antibody control), in order to control the virulence of the strains.

#### Results

[0085]

	A-Homologous		B-Heterologous	
	Rouen (37 kDa) $10^6$ parasites	Rouen (37 kDa) $10^3$ parasites	Weybr. (35 kDa) $10^6$ parasites	Weybr. (35 kDa) $10^3$ parasites
mAb F4.2F8	100	100	100	100
$\alpha$ -Bd17 control	n.d.	30	n.d.	10
isotype control	n.d.	20	n.d.	40
no antibody control	0	20	0	0

Table 3. homologous (A) and heterologous (B) challenge results after passive immunisation

#### Conclusion

homologous challenge results

challenge

#### heterologous challenge results

[0087] As follows from Table 3B, the 37 kDa antibodies do not only provide full protection against homologous challenge (a strain expressing the 37 kDa protein), but also against heterologous challenge (a strain expressing the 35 kDa protein).

Therefore it can be concluded that proteins of the 37 kDa protein family according to the invention induce an antibody response that gives protection against challenge with both homologous *Babesia divergens* strains but also against heterologous strains.

#### Example 5.

#### Synergistic effect of vaccines comprising both a 35 kD protein and a 37 kD protein of the 37 kD protein family

##### Vaccine preparation

[0088] *B. divergens* Rouen/Weybridge parasites were propagated in human erythrocytes in RPMI 1640 medium supplemented with 5 g/l Albumax®. Culture supernatants were harvested at 30-40% parasitaemia, and passed through a 0.22 µm filter. The total volume was kept at 250 µl. One dose of vaccine contained 23.3 µg of Quil A.

##### Immunisation procedure

[0089] Vaccination of gerbils was done with volumina of 250 µl, comprising supernatant of Rouen/Weybridge in a ratio of 0/100, 25/75, 50/50, 75/25 and 100/0. Vaccination was done twice, with a three week interval.

##### Challenge

[0090] Gerbils were challenged intraperitoneally with  $10^3$  or  $10^6$  parasitized erythrocytes/250 µl/gerbil at three weeks after booster injection.

Challenge strain 7107b was passed through gerbils to prepare the challenge inoculum. After developing babesiosis, animals were sacrificed and the level of parasitaemia was determined.

##### Rank score analysis

[0091] Survival, minimal PCV (Packed Cell Volume = haematocrite) and parasite load were submitted to rank score analysis. For each parameter, groups were ranked. The best group was ranked 1, the second best 2 and so on. After ranking the different parameters the total scores for each group were determined. The total net rank score was expressed as the difference between the control and vaccinated groups. This total score can be described as new parameter: protection based upon the different parameters mentioned above.

##### Results

[0092] As can be clearly seen from figure 5, a vaccine based upon a mixture of both 35 kD and 37 kD proteins according to the invention gives a better protection against challenge with both strains comprising 35 kD and 37 kD protein compared to vaccines based upon the same amount of protein from only a 35 kD or a 37 kD protein.

##### Conclusion

[0093] Vaccination with a mixed vaccine comprising both 35 kD and 37 kD protein has a synergistic effect. Such a vaccine protects, when given in low (i.e. below the optimal protective) doses, even better against challenge with both homologous and heterologous strains than comparable low amounts of vaccines based upon pure 35 kD or 37 kD protein.

Legend to the figures

[0094]

Figure 1: Alignment of nucleic acid sequences encoding protein of the 37 kDa protein family in *B. divergens* strains Rouen 1987, Weybridge and Y5, respectively SEQ ID NO: 1, 2 and 3 by Clustal method.

Figure 2: Alignment of amino acid sequences of the 37 kDa protein family in *B. divergens* strains Rouen 1987, Weybridge and Y5, respectively SEQ ID NO: 4, 5 and 6 by Clustal method.

Figure 3: Recombinant proteins of the 37 kD protein family were produced either in a prokaryotic system (A) or in a eukaryotic baculovirus (Bac-to-bac) system (B). The following proteins are visible on gel: purified GST-Bd37 (lane 1) and GST-Bd35 (lane 2) fusion proteins, infected cell lysate containing the Bac-Bd37 protein (lane 3) and non-infected insect cell lysate (lane 4).

Figure 4: Immunoprecipitation of antigens from *B. divergens* Rouen 1987 by sera from gerbils vaccinated with recombinant Bd37 proteins.

Figure 5: Total net rank score of minimal PCV, parasite load and survival of gerbils vaccinated with a mix of Weybridge and Rouen SPA (Soluble Parasite Antigen) challenged with a high or a low challenge dose.



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1. The first step is to identify the main topic of the document. This is often found in the title or the first few paragraphs.

2. The second step is to identify the key points or arguments made by the author. These are usually found in the body of the text.

3. The third step is to identify the evidence or data used to support the arguments. This is often found in the form of tables, figures, or references.

4. The fourth step is to identify the conclusion or final point made by the author. This is usually found in the last few paragraphs.

5. The fifth step is to identify any other important information or details that may be relevant to the topic.

The Ala Thr Val Met Val Lys Thr Leu Arg Val Leu Arg Gly Glu Leu

80                      90                      100

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Arg Gly Gln Arg Ser Ala Thr Leu Ser Glu Ile Ile Lys Ser Asp Gly

            95                  100                      105

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 240                      245                      250

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1. The first part of the paper is devoted to the study of the asymptotic behavior of the solutions of the system (1) for large values of the parameter  $\lambda$ . It is shown that the solutions of the system (1) for large values of  $\lambda$  are asymptotically equivalent to the solutions of the system (2).

[illegible]

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Gln Glu Pro Ala Al Ala Asn Pro Val Val Ser Thr Pro Gly Asn Asp

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185 Lys His Ser Ala Asp Leu Lys Ala Gly Val Glu Leu His Ser Glu Ala  
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1. All acid-forming amino acids are grouped into the acidic group, and the basic amino acids are grouped into the basic group. The acidic amino acids are Aspartic acid (Asp), Glutamic acid (Glu), and Aspartic acid (Asp). The basic amino acids are Lysine (Lys), Arginine (Arg), and Histidine (His).

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QUESTIONS AND ANSWERS

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EP 1 050 541 A1

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15	tta ggt atg aag ggt tat gaa tta gta aag aag ttt aat gag atg aag Leu Gly Val Gln Arg Asp Glu Leu Val Lys Lys Phe Thr Glu Met Ser 205 210 215	670
20	aaa tta tta tta tta tta gaa gat ata tta ggt tta tta aag gta aag Pro Ser Phe Leu Thr Ser Glu Asp Ile Ser Gly Phe Leu Thr Val Pro 220 225 230 235	724
25	gag taa ggt tta tta atg gat gat gat aag tgg aag aat gta gaa aag Glu Tyr Gly Phe Pro Met Asp Ala Ala Lys Trp Lys Lys Val Glu Lys 240 245 250	772
30	aag atg agt gat aag tta gaa tat tta gta ata aca aat gac tta aaa Lys Ile Ser Asp Lys Leu Ala Ser Ser Asp Ile Thr Thr Asp Leu Lys 255 260 265	820
35	aat atg att ata aag tta att gaa aag tat gaa aaa atg atg gat tta Thr Leu Leu Ala Lys Leu Ile Glu Gln Arg Glu Lys Met Met Asp Leu 270 275 280	868
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*Journal of American Studies*, 39 (2005), 1. doi:10.1017/S0021871805005611

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141

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35

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1. 2.

11

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22

40

44

[illegible]

100

[illegible]

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•

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EP 1 050 511 A1

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157 158 159 160

161 Asp Lys Asp Glu Ser Lys Glu Leu Leu Lys Asn Leu Gly Val Gln Arg  
162 163 164 165

166 Asp Glu Leu Val Lys Lys Phe Thr Glu Met Ser Pro Ser Phe Leu Thr  
167 168 169

170 Ser Glu Asp Ile Ser Gly Phe Leu Thr Val Pro Glu Tyr Gly Phe Pro  
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176 177 178

179 Leu Glu Ser Ser Asp Ile Thr Thr Asp Leu Lys Thr Leu Leu Ala Lys  
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184 185 186

187 Gly His Glu Asp Lys Pro Ala Arg Ser Gly Gln Gly Ser Gly Pro Lys  
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 02230 PRIMER Bd37 low

aaaggatcca aaagctacat agctgtccat t

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<213> Bacteriophage T4

02200  
 02201 END OF LINE  
 02202 CHAIN TO  
 02203 PRIMER BEG. W/

$\frac{1}{\sqrt{2}} \begin{pmatrix} 1 & -i \\ 0 & 1 \end{pmatrix}$

## Claims

1. Nucleic acid sequence of *Bat-esia divergens* characterised in that it encodes a protein of the 37 kD protein family or an immunogenic fragment thereof

10. The weight of a 1000-kg car is 9800 N. The car is supported by four wheels. Assuming the weight is distributed equally on the four wheels, how much force does each wheel exert on the road?

5. Nucleic acid sequence according to claim 5, characterised in that it comprises the sequence shown in SEQ ID NO 1 or 2.
6. Nucleic acid sequence encoding a protein of the 37 kD protein family that is capable of binding to the monoclonal antibody F4/5 B.
7. Recombinant DNA molecule characterised in that it comprises a nucleic acid sequence according to claims 1-6 and regulating sequences enabling expression of said nucleic acid sequence.
8. Live recombinant carrier micro-organism comprising a nucleic acid sequence according to claims 1-7.
9. Live recombinant carrier according to claim 8, characterised in that said carrier additionally comprises a heterologous gene encoding an antigen of a cattle pathogen, which is able to elicit an immune response.
10. Live recombinant carrier according to claim 9, characterised in that said cattle pathogen is selected from the group of cattle pathogens, consisting of Bovine Herpesvirus, bovine Viral Diarrhoea virus, Parainfluenza type 3 virus, Bovine Paramyxovirus, Foot and Mouth Disease virus, *Pasteurella haemolytica*, Bovine Respiratory Syncytial Virus, *Theileria parva*, *Theileria annulata*, *Babesia bovis*, *Babesia bigemina*, *Babesia major*, *Trypanosoma* species, *Anaplasma marginale*, *Anaplasma centrale* or *Neospora caninum*.
11. Host cell comprising a nucleic acid sequence according to claims 1-6, a recombinant DNA molecule according to claim 7 or a live recombinant carrier according to claims 8-10.
12. Vaccine inducing protection against infection of cattle with *B. divergens* or the clinical manifestations of the infection, characterised in that it comprises at least one protein of the 37 kD protein family or an immunogenic fragment thereof and a pharmaceutically acceptable carrier.
13. Vaccine according to claim 12, characterised in that it comprises two or more different proteins of the 37 kD protein family.
14. Vaccine according to claim 13, characterised in that it comprises 50-75% of a 37 kD member of the 37 kD protein family and 50-25% of a 35 kD protein member of the 37 kD protein family.
15. Vaccine inducing protection against infection of cattle with *B. divergens* or the clinical manifestations of the infection, characterised in that it comprises a nucleic acid sequence according to claims 1-6, a recombinant DNA molecule according to claim 7, a live recombinant carrier according to claims 8-10 or a host cell according to claim 11.
16. Vaccine protecting against infection of cattle with *B. divergens* or the clinical manifestations of the infection, characterised in that it comprises antibodies against a protein of the 37 kD family.
17. Method for the preparation of a vaccine according to claim 12, 15 or 16, characterised in that said method comprises the admixing of a protein of the 37 kD protein family or an immunogenic fragment thereof, antibodies against a protein of the 37 kD family, a nucleic acid sequence according to claims 1-6, a recombinant DNA molecule according to claim 7, a live recombinant carrier according to claims 8-10 or a host cell according to claim 11 and a pharmaceutically acceptable carrier.
18. Method for the preparation of a vaccine according to claim 13, characterised in that said method comprises the admixing of two different proteins of the 37 kD protein family or immunogenic fragments thereof and a pharmaceutically acceptable carrier.
19. Diagnostic kit for the detection of antibodies against *Babesia divergens*, said kit comprising purified protein of the

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21. Method for the detection of antibodies against *Babesia divergens*, said method comprising incubating serum with a protein of the 37 kD protein family or an antigenic fragment thereof
22. Method for the detection of *Babesia divergens* parasites, said method comprising incubating serum with antibodies against a protein of the 37 kD protein family
23. Use of a protein of the 37 kD protein family or a mixture of such proteins for the manufacture of a vaccine inducing protection against infection of cattle with *B. divergens* or the clinical manifestations of the infection



```

      100      200      300      400      500
      |         |         |         |         |
1  GAATTC CGACCATACGAATAATTATGTTGTGGTTTAACCTATTAATATCCTAATCAAG
2  GAATTC CGACCATACGAATAGTTATTTTCTTTTAACCTATTAATATCCTAATCAAG
3  -----CAAG
      |         |         |         |         |
      60      70      80      90     100     110
      |         |         |         |         |
1  TTCTTT TAAGTACGATGAAAACCACTAAGATTCTCAACACTGCTGCTATCTGCCTCCTG
2  TTCTCT TAAGTACGATGAAAACCACTAAGATTCTCAACACTGCTGCTATCTGCCTCCTG
3  GATCCTC TAAGTACGATGAAAACCACTAAAATTCTCAACACGGCTGCTATCTGCCTCCTG
      |         |         |         |         |
      120     130     140     150     160     170     180
      |         |         |         |         |         |         |
1  GCTATG GTTTCAATGGCAATAATGTGAGCTGCACCAATCTCAATGGCTCACAGGAGCCA
2  GTATAG GTTTCAATGGCAATAATGTGCGCTGCACCAATCTCAGTGGCGGCACAGCAGCCA
3  GTATAG GTTTCAATGGCAATAATGTGAGCTGGCGCATATCAGTGGCACACAGGAGACA
      |         |         |         |         |         |         |
      190     200     210     220     230     240
      |         |         |         |         |         |         |
1  TAACTTTTCTTTTAACTGTTTGAATGATGGTACGATGGCTGTATCAATGAA
2  TAACTTTTCTTTTAACTGTTTGAATGATGGTACGATGGCTGTATCAATGAA
3  TAACTTTTCTTTTAACTGTTTGAATGATGGTACGATGGCTGTATCAATGAA
      |         |         |         |         |         |         |
      250     260     270     280     290     300
      |         |         |         |         |         |         |
1  TAACTTTTCTTTTAACTGTTTGAATGATGGTACGATGGCTGTATCAATGAA
2  TAACTTTTCTTTTAACTGTTTGAATGATGGTACGATGGCTGTATCAATGAA
3  TAACTTTTCTTTTAACTGTTTGAATGATGGTACGATGGCTGTATCAATGAA

```

310 320 330 340 350 360  
 1 AAAATCTTTTAAAGGTTTGTGTAAGAAATCTAGATCTGCTCATGCGGGAATCAGCGGG  
 2 GAAAATCTTTAAAGGTTTGTGTAAGAAATCTAGATCAACTCCGTGGGGAATCAGCGGG  
 3 ---AGCTCTGTAAAGGTTTGTGTAAGAAATCTGAGGATTTACGTGAAGAGCTTAAGGT  
 .....

370 380 390 400 410 420  
 1 CAGCTTAAAGGTTTCTTTTCAGAGATAATTAATCGGATGCTCCATTCACTATTTTCAG  
 2 CAGCTTAAAGGTTTCTTTTCAGAGATAATTAATCGGATGCTCCATTCACTATTTTCAG  
 3 CAGCTTAAAGCTTCTTTTCAGAGATAATTAATGAGTCCGATGAGGATTTCCGTTTCTTCAA  
 .....

430 440 450 460 470 480  
 1 TTGCTTGGCTACCTTCGTGTTGTGACACAGATCTTCTCCTGAAAGTTGATTCACGSAAG  
 2 TTGCTTGGCTACCTTCGTGTTATCGACACAGATCTTCTCCTGAAGGTTGATTCGGTGAAG  
 3 TTGATTTATTCCTTCGTGTTATTGACACAGATCTTCTCCTGAAGGTTGACGGGACAAAG  
 .....

490 500 510 520 530 540  
 1 GTTGATGAAGCGGCAAGAAAGGTCAGGGCTACCTTGAAAAAATTGGAATAAGGGGTGAC  
 2 GTTATTCAGAGCGGCAAGAAAGGTCAGAGCTACCTTGAAAAAATTGGAATAAGGGGTGAC  
 3 GTTAAAGAAAGGCAAGTAAAGTCAAGGGCTACCTTGAGTCTATTGGAATAAGGGGTGGA  
 .....

550 560 570 580 590 600  
 1 AATCTTGAAGGCTTTTAAATTTATATAAAGTTTATGAAATCAATAAGTTACT  
 2 AATCTTGAAGGCTTTTAAATTTATATAAAGTTTATGAAATCAATAAGTTACT  
 3 AATCTTGAAGGCTTTTAAATTTATATAAAGTTTATGAAATCAATAAGTTACT  
 .....

610 620 630 640 650 660  
 1 ATGAAATCTTAAAGGCTTAAAGGCTTAAAGGCTTAAAGGCTTAAAGGCTTAAAGGCTT  
 .....

AATGAAATCTTAACTTAAAGAAACCTATAGTGAAACCAAGGCGG---GTGAGGC  
 AATGAAATCTTAACTTAACTTAAAGAAACCTATAGTGAAACCAAGGCGG---GTGAGGC  
 AATGAAATCTTAACTTAAAGTAAAGAAACCTATAGTGAAACCAAGGCGG---GTGAGGC

|   |                                                               |       |       |       |       |       |
|---|---------------------------------------------------------------|-------|-------|-------|-------|-------|
|   | 730                                                           | 740   | 750   | 760   | 770   | 780   |
|   |                                                               |       |       |       |       |       |
| 2 | TTGCTT CTAGCATGAAGACGAGCATGATGAAC TACTTAAGAAGTTTGCTGCCC TTAC  |       |       |       |       |       |
| 3 | TTGCTT CTAGCATGAAGACGAGCATGAGGAAC TAC TTAAGAAGTTTGCTGCCC TTAA |       |       |       |       |       |
| 4 | TTACTGAGAGAATTTCCTGCTGCAAGCTGATGAATTGCTCAAGAAGTTTACTCAGATGAGC |       |       |       |       |       |
|   | .....                                                         | ..... | ..... | ..... | ..... | ..... |

```

      800      810      820      830      840
      |      |      |      |      |
1  CCTAAGT TCCTTAAGCTTAGAGGATATATCTGGCTACCTTACCGTGCCCGGAATACGGTGGC
.  CCTACT TCCTTATCGAAGAGAGGATATATCTGGCTACCTTACCGTGCCCGGAATACGGTGGC
.  GGATCG TCCTTGAGCTTAGAAGATATATCTGGCTTCTTCACSGTGCCCGGAGTACGGTTTC
      *      *      *      *      *      *      *      *      *      *

```

```

      880      890      900
      |      |      |      |      |
1  TTTATTTATCTGTGGAAGTGTGAAAA GTGTGGAAGGAATGATCCATGGAAAAGCTCGAGTCT
2  TTTATTTATCTGTGTGGAAGTGTGAAAA GTGTGGAAGGAAAGATCAATGAAAAGCTCGAGTCT
3  TTTATTTATCTGTGTGGAAGTGTGAAAA GTGTGGAAGGAGAGATCAATGATAAGCTCGAGTCT
      * * * * *

```

[illegible]

```

1 ATGATG AATTTGCTATACGGCCCTATTGGTCATCAGGATTGTGCTGCAGGATCAGGTCAG
2 ATGATG AATTTGCTATACGGCCCTATTGGTCATCAGGATTGTGCTGCAGGATCAGGTCAG
3 ATGATG AATTTGCTATACGGCCCTATTGGTCATCAGGATTGTGCTGCAGGATCAGGTCAG
.....

```

```

      1030      1040      1050      1060      1070      1080
      |          |          |          |          |          |

```

```

1 GGATCT AGTCCTAAGAAGCCATCCTTGGCTGCTGTACCTTCTTCTTTGTCTGCCATTGTC
2 GGATCT AGTCCTAAGAAGCCATCCTTGGCTGCTGTACCTTCTTCTTTGTCTGCCATTGTC
3 GGATCT AGTCCTAAGAAGCCATCCTTGGCTGCTGTACCTTCTTCTTTGTCTGCCATTGTC
.....

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```

      1090      1100      1110      1120      1130      1137
      |          |          |          |          |          |

```

```

1 TTCGGT ATCAATTGTATCAATGTTCTAAGTGGACAGCTATGTAGCTTTTGGATCCTGT
2 TTCGGT ATCAATTGTATCAATGTTCTAAGTGGACAGCTATGTAGCTTTTGGATCCTGT
3 TTCGGT ATCAATTGTATCAATGTTCTAAGTGGACAGCTATGTAGCTTTTGGATCCTGT
.....

```

1. Rohm 1984

2. Wepf 1984

3. Wepf

Fig. 2 :

```

      10      20      30      40      50      60
      |      |      |      |      |
4  ME T L E N T A A T L L A M S E N G N I V E C I N L G S Q E P A A A N P V V S T P G N D A Q Q A G T Q Q G G A N
5  M F I S F I L L A A T L L A M S E N G N I V C T H I L G A Q Q I A A S T P G V E T S G N D S Q Q G I T S - - - -
6  M E T L E N T A A T L L A M S E N G N I V C A R I T Q T Q L T A A N S G D E T T R N D A Q Q S G E V - - - -
   .....

      70      80      90     100     110     120
      |      |      |      |      |      |
4  E K V I T Q Q I Q D A A G E I T A T V V V F T L D V I R G E L R G Q R E A F L S E I I K S D G P F T I L Q L V G Y L R
5  - - - - - Q Q P Q Q A A G E T F A K V V V F T L D E L R G E L R G Q R E A F L S E I I K S D G P F T I L Q L V G Y L R
6  - - - - - Q Q T F Q Q T V K A V V E S E D I R E E L R G Q R E T F L S K L I E S D G A F G F L Q L I D F L R
   .....

     130     140     150     160     170     180
     |      |      |      |      |      |
4  V V D T L L L K V D S T K V D E A G K F V F A Y L E K I G I R G D S V E A A L D N L M I K V Y E I T K G T V E S S A Q
5  V I T L L L I K V D S V K V T E A G K F V F T Y L E E I C I T G D S V E A A L D N L M I K I Y E L T N G T V E S S P Q
6  V I T L L L L K V D S T K V E A G V F V F A Y L E S I G I K G S V E E C L D N L M T K V S A I T R G T V E G S A Q
   .....

     190     200     210     220     230     240
     |      |      |      |      |      |
4  M I T F L E X T I L L F F S E D L R A E Q E L H S E A R G G F A L L S S M E T Q H D E L L R F F A A L T P T F L T S E
5  L I F F A L P T L I I F F P E D L R A E Q E L H S E A R G G F A L L S S M E T Q H D E L L R F F A A L N P T F L S T E
6  M I T F L E X T I L L F F S F I R A E Q E F G D F L E S K E L L K N L S V Q R D E L V R K F T E M S F S F L T S F
   .....

      250     260     270     280     290     300
      |      |      |      |      |      |
4  E I L L L L V I L Y G A I M N A A K W E R V E M I R G E L E S E V E A N L F A L V A E L I K L R E Q M M D I L Y G
5  E I L L L L V I L Y G A I M N A A K W E R V E I R I N F L E S E I Q S D L R A L V A E L I K L R E Q M M D I L Y G
6  E I L L L L V I L Y G I M A A K W E R V E K K I S I E L E S S D I T T L K T I L A K L I F O R E K M M D I L Y G
   .....

```

```

4      FIGHHDC AAGSGQGS SPKKPSFAAVPSSL SAIVFGIIVSMF
5      FIGHHDC AAGSGQGS SPKKPSFAAVPSSL CAIVFGIIVSMF
6      FIGHEDC PARSGQGS GPKEPSFAAVPSSL CAIVFGIIVSMF
      .....

```

```

4: Strain Rouen 1987      341 aa
5: Strain Weybridge      331 aa
6: Strain Y5              321 aa

```

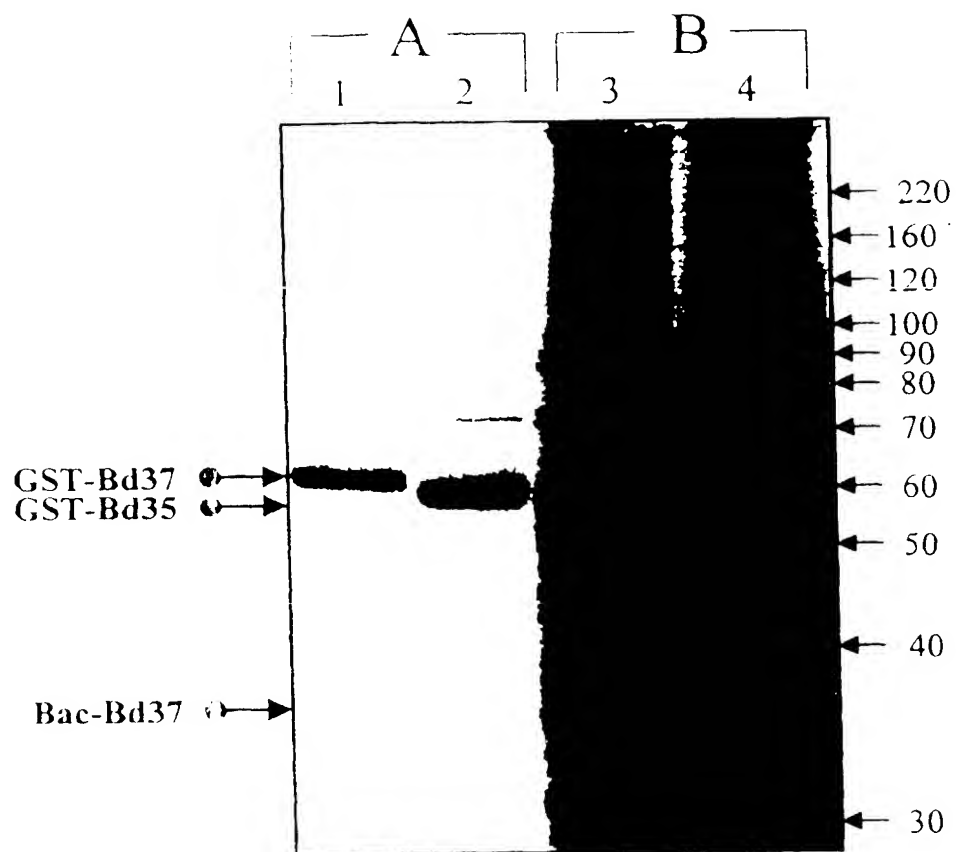


Fig.3

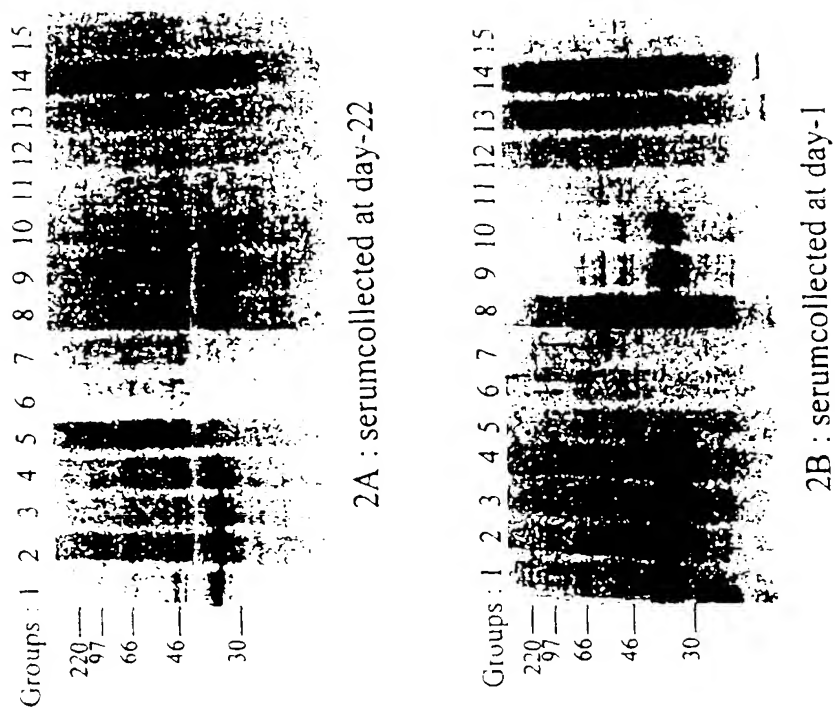


Fig. 4



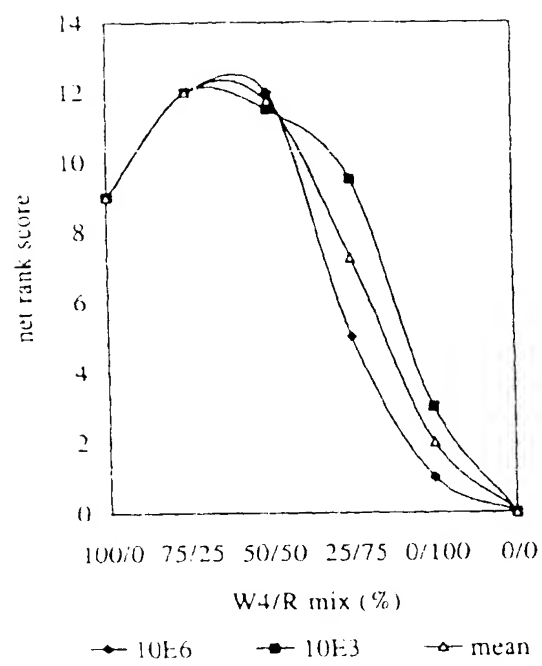


Figure 5